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The DNA translocase RAD5A acts independently of the other main DNA repair pathways and requires both its ATPase and RING domain for activity in *Arabidopsis thaliana*

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SUMMARY

Multiple pathways exist to repair DNA damage induced by methylating and cross-linking agents in *Arabidopsis thaliana*. The SWI2/SNF2 translocase RAD5A, the functional homolog of budding yeast Rad5 that is required for the error-free branch of post replicative repair, plays a surprisingly prominent role in the repair of both kinds of lesions in Arabidopsis. Here we show that both the ATPase domain and the ubiquitination function of the RING domain of the Arabidopsis protein are essential for the cellular response to different forms of DNA damage. To define the exact role of RAD5A within the complex network of DNA repair pathways, we crossed the *rad5a* mutant line with mutants of different known repair factors of Arabidopsis. We had previously shown that RAD5A acts independently of two main pathways of replication-associated DNA repair defined by the helicase RECQ4A and the endonuclease MUS81. The enhanced sensitivity of all double mutants tested in this study indicates that the repair of damaged DNA by RAD5A also occurs independent of nucleotide excision repair (AtRAD1), single-strand break repair (AtPARP1), as well as micro-homology mediated double-strand break repair (AtTEB). Moreover, RAD5A can partially complement for a deficient AtATM-mediated DNA damage response in plants, as the double mutant shows phenotypic growth defects.

INTRODUCTION

Cells possess different mechanisms to facilitate the removal of DNA adducts and to overcome replication-blocking lesions. There are different kinds of DNA damage, such as methylated bases and DNA cross-links (CLs), which are repaired by more than one pathway with varying degrees of efficiency. Genotoxic agents can distinctively trigger these pathways: Mitomycin C (MMC) mainly forms interstrand CLs on DNA (Rink *et al.*, 1996), whereas the structures preferentially formed by cisplatin are intrastrand CLs (Eastman, 1985). Methylated bases are generated by methyl methanesulfonate (MMS) treatment (Pegg, 1984). DNA repair pathways that deal with such kinds of damage include base excision repair (BER), nucleotide excision repair (NER) and post replicative repair (PRR) (Schröpfer *et al.*, 2014a). These pathways involve different DNA repair proteins with characteristic sets of functional domains, depending on their roles within their respective pathways.

In yeast, PRR consists of two branches: an error free branch and an error prone branch that is also known as translesion synthesis (TLS). TLS involves low-fidelity polymerases such as Polη and Polζ (Broomfield *et al.*, 2001). The error free branch requires the Ubc13/Mms2/Rad5 complex to bypass replication fork blockage, probably via template switch mechanisms (Barbour and Xiao, 2003). This branch is induced through polyubiquitination of proliferating cell nuclear antigen (PCNA), in which Rad5 functions as an E3 ubiquitin ligase. Furthermore, Rad5 seems to be mechanistically involved within the repair process itself through its DNA translocase activity. By regressing the replication fork, a so-called chicken foot structure is formed that allows the blocked strand to be further

elongated, using the newly synthesized strand of the parental strand as a template. The fork regression activity of Rad5 has been shown *in vitro* (Blastyak *et al.*, 2007).

Two human orthologs, HLTF and SHPRH, have been identified and have both been proposed to be tumor suppressor proteins (Motegi *et al.*, 2008; Unk *et al.*, 2010). They have been shown to act in non-redundant, damage-specific responses to UV- or MMS-induced mutagenesis (Lin *et al.*, 2011). Resembling further their homologs in yeast, both are able to turn monoubiquitinated PCNA into a polyubiquitinated form *in vitro* (Unk *et al.*, 2006; Unk *et al.*, 2008). All findings indicate that HLTF and SHPRH are crucial for genome maintenance in response to DNA damage.

In former studies, we have shown that AtRAD5A, the functional homolog of ScRad5 in *A. thaliana*, is involved in the repair of various genotoxic lesions (Chen *et al.*, 2008). The two branch PRR model also seems to apply for Arabidopsis, where AtREV3 is a known factor within plant TLS and a *rad5a rev3* double mutant displayed an additive sensitivity to the replication blocking DNA damage of MMS (Sakamoto *et al.*, 2003; Wang *et al.*, 2011; Kobbe *et al.*, 2015). Furthermore, RAD5A seems to be involved in the synthesis-dependent strand annealing (SDSA) mechanism of double-strand break (DSB)-induced homologous recombination (HR) (Chen *et al.*, 2008; Mannuss *et al.*, 2010). Previously, we were able to define the RAD5A-dependent pathway in a broader context by investigating the *rad5a recq4a* and *rad5a mus81* double mutants (Mannuss *et al.*, 2010). We found that the DNA helicase RECQ4A and the endonuclease MUS81 characterize parallel pathways to RAD5A that are involved in processing different DNA intermediates during replication-associated DNA repair (Mannuss *et al.*, 2010). More recently, we could demonstrate *in vitro* that RAD5A is a DNA translocase that is able to catalyze fork regression and branch migration of

Holliday junctions. Interestingly, the protein harbors a conserved HIRAN domain, the presence of which is essential for DNA repair *in vivo* and required for DNA repair intermediate binding *in vitro* (Kobbe *et al.*, 2016). Besides the HIRAN domain, AtRAD5A harbors two additional conserved domains, the functions of which have not been tested in DNA repair *in planta*: The C-terminal half of the protein contains a helicase domain, subdivided into 7 conserved motifs. Motif I is also referred to as Walker A box, motif II as Walker B box. As a further characteristic feature of the SNF2-protein family, a RING domain is embedded within motif III and IV of the helicase domain. RING domains represent a specialized type of zinc finger, in which cysteine or histidine residues coordinate two Zn^{2+} ions. Functions of RING domains include mediation of protein-protein binding and are typically found in proteins that function as E3 ubiquitin ligases (Lorick *et al.*, 1999). Replacing one of the Zn^{2+}-coordinating cysteines in ScRad5 inhibits its interaction with Ubc13, which forms an E2 complex with Mms2 in yeast (Ulrich and Jentsch, 2000; Ulrich, 2003). Mutating the RING domain in that manner also leads to increased UV sensitivity in the respective yeast mutant (Ulrich, 2003).

Due to the constant exposure of plants to UV light, the excision repair pathway involving RAD1 is of particular importance. RAD1 (also known as UVH1 or XPF) forms a heterodimeric endonuclease complex together with ERCC1 (also known as RAD10). The Arabidopsis ortholog of RAD1 has been shown to be involved in numerous aspects of DNA maintenance, including NER (Fidantsef *et al.*, 2000), coinciding with the roles of the yeast and mammalian orthologs that have been extensively studied (Aboussekhra and Wood, 1994; de Laat *et al.*, 1999). AtRAD1 functions within the so-called "dark" NER pathway in response to UV-induced DNA damage that is not repaired by light-activated photolyases (Liu *et al.*, 2000).

At*rad1* antisense lines showed sensitivity towards UV and the cross-linking agent MMC (Gallego *et al.*, 2000). Furthermore, AtRAD1 is required for homologous recombination in the presence of non-homologous overhangs (Dubest *et al.*, 2002).

Poly ADP-ribose polymerases (PARPs) use NAD^+ as a substrate to catalyze the synthesis and transfer of poly ADP-ribose (PAR) to target proteins (Woodhouse and Dianov, 2008). In animals, PARP1 has been shown to be involved in DNA single-strand break repair (SSBR) and BER (Schreiber *et al.*, 2002; Masaoka *et al.*, 2009). The functions of the PARP1 ortholog in Arabidopsis include a role within stress tolerance, programmed cell death and backup non-homologous end-joining (B-NHEJ) (Amor *et al.*, 1998; de Block *et al.*, 2005; Vanderauwera *et al.*, 2007; Jia *et al.*, 2013). Furthermore, PARP1 seems to be involved within the repair of base alterations, due to a *parp1* mutant displaying increased sensitivity towards MMS-induced DNA damage (Boltz *et al.*, 2014).

AtTEBICHI (TEB) is encoded by the *TEB* gene and has been identified as the homolog of the mammalian DNA polymerase θ (POLθ), with widespread functions within the maintenance of genome stability (Inagaki *et al.*, 2006). POLθ plays an important role in the repair of DNA DSBs, more precisely in microhomology-mediated end-joining (MMEJ) (Muzzini *et al.*, 2008; Roerink *et al.*, 2014; Ceccaldi *et al.*, 2015; Kent *et al.*, 2015; Mateos-Gomez *et al.*, 2015; van Schendel *et al.*, 2015). POLθ has been identified as a novel druggable target for cancer therapy, since a synthetically lethal relationship between the HR pathway and POLθ-mediated repair was revealed (Ceccaldi *et al.*, 2015). With its helicase and DNA polymerase domains, TEB also seems to be involved in cell division and differentiation, with At*teb* mutants showing various defects in plant morphology resembling a fasciated phenotype, to a certain extent (Inagaki *et al.*, 2006). Furthermore, *teb* mutants are sensitive towards the

cross-linking and methylating agents MMC and MMS, respectively, and show reduced levels of intrachromosomal recombination (Inagaki *et al.*, 2006). By assessing double mutants deficient in TEB and other factors involved in both homologous recombination (HR) and cell cycle progression, it has been shown that TEB functions within the progression of DNA replication and correct gene expression during development (Inagaki *et al.*, 2009). Recently it was reported that the function of TEB is essential for T-DNA integration (van Kregten *et al.*, 2016).

The two protein kinases Ataxia teleangiectasia mutated (ATM) and ATM and Rad3-related (ATR) are the central regulators of DNA damage response in mammals. Whereas ATM primarily mediates the response to DSBs, ATR is activated by single-stranded DNA and stalled replication forks (Bensimon *et al.*, 2011; Flynn and Zou, 2011). ATM deficiency in humans is associated with the Louis-Bar syndrome and heterozygous mutations lead to an enhanced predisposition to cancer (Savitsky *et al.*, 1995). Orthologs of ATM and ATR have also been identified in Arabidopsis. At*atm* as well as At*atr* null mutants are viable, even though At*atm* lines show reduced fertility and the *atm atr* double mutant is completely sterile (Culligan *et al.*, 2004; Culligan *et al.*, 2006; Culligan and Britt, 2008). Furthermore, the At*atm* mutant is sensitive towards ionizing radiation and the methylating agent MMS (Garcia *et al.*, 2003). It was shown that the transcriptional induction of DNA repair genes after treatment with ionizing radiation is impaired in At*atm* mutants, which confirms the central role of ATM in DNA damage response in Arabidopsis (Ricaud *et al.*, 2007).

Using mutational analysis, in the present study, we demonstrate that the helicase domain as well as the RING domain are essential for the function of RAD5A in DNA repair. Further

defining the role of RAD5A in the complex network of DNA repair factors, we present evidence that RAD5A acts independently of various other well-known DNA repair pathways.

RESULTS

The helicase and RING domains are essential for RAD5A function

To define the role of the enzymatic domains of the protein RAD5A in DNA repair *in planta*, analysis of hypersensitivity of *rad5a-2* mutant lines transformed with different *RAD5A* gDNA constructs was performed. In *Saccharomyces cerevisiae*, point mutations KT538/539AA in Sc*Rad5* have previously been described in order to abolish all ATP-binding and hydrolytic activities of the protein (Richmond and Peterson, 1996). A previously characterized mutation in the ScRad5 RING domain (C914S), that affects one of the cysteines involved in Zn^{2+} coordination, had been shown to disrupt interaction with the E2 enzyme (Ulrich and Jentsch, 2000). We identified the respective conserved amino acids in AtRAD5A and produced the constructs *RAD5A-HD* (KT425/426AA) and *RAD5A-RD* (C794S) with the respective amino acid exchanges via mutagenesis. (Figure 1). *In vitro* analysis of RAD5A expressed in *E. coli* demonstrated that the (KT425/426AA) mutated protein (*RAD5A-HD*), in contrast to the wild type protein, is indeed devoid of ATPase activity (Kobbe *et al.*, 2016).

The *rad5a-2* mutant is hypersensitive to treatment with the DNA cross-linking agents cisplatin and MMC, as well as the base-methylating agent MMS (Chen *et al.*, 2008). After treatment with 5 µg/ml of the interstrand CL-inducing agent MMC, the *rad5a-2* mutant displayed a strong growth impairment with a tenth reduced growth compared to that achieved for wild type (WT) (Figure 2a). We confirmed that the sensitivity of *rad5a-2* against treatment with MMS, MMC and cisplatin is due to the mutation of the *RAD5A* gene by

complementation using a T-DNA construct containing *RAD5A* gDNA flanked by the natural promoter and terminator. Since the construct was transformed via *Agrobacteria,* and therefore integrated at random, we tested randomly selected independent integration lines to exclude position effects. *rad5a-2* mutant lines transformed with the *RAD5A* WT construct (Figure 2a) exhibited a relative dry weight comparable to WT. The full complementation of the *rad5a* phenotype transformed with the *RAD5A* WT construct indicates that the cloned promoter and terminator regions result in an appropriate level and pattern of expression. However, *rad5a-2* mutant lines expressing the *RAD5A-HD* or *RAD5A-RD* construct showed hypersensitivity when compared to the WT (Figure 2b, c). Thus, the hypersensitivity of *rad5a-2* could not be complemented by the expression of the helicase- or RING-defective *RAD5A-HD* or *RAD5A-RD* variant, respectively. Similar results were obtained after treating the same lines with the intrastrand CL-inducing agent cisplatin (Figure 2d-f) and the methylating agent MMS (Figure 2g-i).

As a control experiment, WT lines were transformed with the identical constructs, full length RAD5A, *RAD5A-HD* and *RAD5A-RD.* The relative dry weight of these lines did not differ from the WT after treatment with MMC (Supplemental figure S1). These results exclude a possible negative complementation effect on the sensitivity of plants. To verify the genuine expression of the RAD5A transgene in the transformed *rad5a-2* lines, real-time PCR expression analysis was performed and revealed similar or slightly higher levels of expression compared to the WT (Col-0) (Supplemental figure S2). Presence of the different protein variants was furthermore confirmed via western blotting using a RAD5A antibody (Kobbe *et al.*, 2016) (Supplemental figure S3). This analysis also confirmed the absence of RAD5A protein in *rad5a-2* mutants.

In summary, these data demonstrate that the helicase, as well as the E3 ubiquitin ligase activity of RAD5A, are essential for its function in repairing different kinds of DNA CLs and methylated DNA.

RAD5A and RAD1 have independent roles in the repair of DNA cross-links and base methylations

Not all factors involved in either intra- and interstrand CL repair or the repair of methylated bases are also involved in the repair of the other two lesions. Subsequently, to gain an increased understanding of the role of RAD5A in DNA repair, it was of special interest to define whether RAD5A fulfils its function in collaboration with other prominent DNA repair pathways in plants.

AtRAD1, which is part of a heterodimeric endonuclease complex with AtRAD10, is a prominent factor of NER in Arabidopsis (Fidantsef *et al.*, 2000). Consequently, the mutant is sensitive to a variety of DNA damaging agents.

To further define the role of RAD1 in DNA repair in respect to RAD5A, we created the double mutant *rad5a-2 rad1-1* by crossing the respective homozygous single mutant lines. A double mutant, homozygous for *rad5a-2* and *rad1-1*, was identified in the F2 generation via PCR-based genotyping. The plants showed no difference to the WT phenotype regarding viability or development, under standard growth conditions. To characterize the sensitivity of *rad5a-2 rad1-1,* we determined the dry weight of the double mutant, the respective single mutants and the WT using sensitivity assays as described after induction of base methylations by MMS and induction of interstrand and intrastrand CLs by MMC and cisplatin, respectively.

Both single mutants *rad5a-2* and *rad1-1* showed hypersensitivity towards MMS. At a concentration of 100 ppm MMS (Figure 3b), the double mutant exhibited a strong additive hypersensitivity, revealed by a relative dry weight that was significantly lower compared to either single mutant. Thus, using an adequate concentration of MMS, we were able to show that RAD1 is involved in the repair of MMS-induced DNA damage, in a RAD5A independent manner.

In response to the CL-inducing agents MMC and cisplatin, the *rad1-1* single mutant exhibited a severe hypersensitivity to concentrations that did not affect the *rad5a-2* mutant, which we previously demonstrated to be sensitive towards CLs (Chen *et al.*, 2008) (Figure 3c, e). However, the double mutant *rad5a-2 rad1-1* showed a level of hypersensitivity that was significantly greater than the sensitivity of *rad1-1*. At slightly higher concentrations, RAD1 deficient plants were lethally damaged and therefore no synergistic effects could be measured, even though the *rad5a-2* single mutant exhibited a significant reduction of growth in comparison to the WT (Figure 3d, f). Taken together, these results indicate that RAD1 is involved in a very critical step of CL repair and acts independently from RAD5A.

RAD5A and PARP1 have independent roles in the repair of base methylations

AtPARP1 (also known as ZAP, At2g31320) is the Arabidopsis homolog of HsPARP1, which is well studied due to its involvement in SSBR and BER (Schreiber *et al.*, 2002; Masaoka *et al.*, 2009).

In an attempt to define the role of RAD5A in relation to PARP1, whose respective mutants are known to be sensitive towards MMS-induced DNA damage, we created the double mutant *rad5a-2 parp1-1*. The double mutant was viable and displayed no obvious

differences in phenotype to the WT. To characterize the sensitivity of *rad5a-2 parp1-1*, we determined the dry weight of the double mutant, the respective single mutants and the WT using sensitivity assays with MMS, MMC and cisplatin, as previously described.

In response to the CL-inducing agents MMC and cisplatin, the *parp1-1* mutant did not show hypersensitivity and the *rad5a-2 parp1-1* double mutant was comparable to the *rad5a-2* single mutant at all tested concentrations of the DNA cross-linkers (Supplemental Figure S4). However, after inducing base methylations via MMS, both single mutants displayed a significant hypersensitivity compared to the WT, at one concentration at least (Figure 4). The double mutant *rad5a-2 parp1-1* exhibited an additive sensitivity at both tested concentrations of MMS that was significantly higher compared to the respective single mutants. Thus, AtPARP1 seems to be involved into the repair of base alkylations, but not DNA CLs, and thereby acts in a parallel pathway to AtRAD5A.

Absence of RAD5A and TEBICHI results in an additive sensitivity towards DNA cross-links and base methylations

AtTEB is the homolog of the mammalian DNA polymerase θ. Pol θ has been reported to be involved in tolerance to DNA damage and DSB repair in several multicellular eukaryotes (Boyd *et al.*, 1990; Ukai *et al.*, 2006; Roerink *et al.*, 2014; Ceccaldi *et al.*, 2015; Kent *et al.*, 2015; Mateos-Gomez *et al.*, 2015; van Schendel *et al.*, 2015). In Arabidopsis, due to the sensitivity of the respective *teb* mutant towards MMC and MMS, TEB could also be linked to DNA repair (Inagaki *et al.*, 2006). Even further, TEB seems to be required for efficient intrachromosomal DNA recombination, cell differentiation, cell cycle control and progression of replication (Inagaki *et al.*, 2006; Inagaki *et al.*, 2009). We were intrigued by

the variety of sensitivities and features of *teb* mutants, which prompted us to create a *rad5a teb* double mutant to further define the affected pathways.

This double mutant displayed morphological defects such as serrated leaves and fasciation, under standard growth conditions, as reported for all TEB deficient plant lines (Inagaki *et al.*, 2006). To characterize the sensitivity of *rad5a-2 teb-5,* we determined the dry weight of the double mutant, the respective single mutants and the WT using sensitivity assays as described after induction of base methylations by MMS and inducing interstrand and intrastrand CLs by MMC and cisplatin, respectively.

Both single mutants *rad5a-2* and *teb-5* displayed hypersensitivity towards MMS treatment in comparison to the WT plants. The relative dry weight of the single mutants was reduced to a half and a third of the untreated controls, after induction with 75 ppm MMS (Figure 5a). At this concentration, the *rad5a-2 teb-5* double mutant showed an additive hypersensitivity that was significantly higher than the level of either single mutant. Due to the extreme hypersensitivity of TEB deficient plant lines towards MMS, this additive effect was not seen any more at higher concentrations, which resulted in the death of both single and double mutant plants (Figure 5b). Thus, RAD5A and TEB play different roles within the repair of MMS-induced DNA damage and act in parallel pathways.

After induction of DNA CLs by MMC and cisplatin, both single mutants *rad5a-2* and *teb-5* exhibited a significant reduction of the relative dry weight compared to the WT (Figure 5c-f). After treatment with 1.5 µg/ml MMC (Figure 5c), 1.5 µM and 2.5 µM cisplatin (Figure 5e, f), an additive hypersensitivity of the *rad5a-2 teb-5* double mutant could be observed, indicated by a reduced dry weight that was significantly below the level of both single mutants. Treatment with 2.5 µg/ml MMC revealed no additive effect, due to the severe

sensitivity of the RAD5A deficient plant lines. Consequently, these results indicate that RAD5A and TEB are involved in the repair of inter- and intrastrand CLs, acting within independent pathways.

The *rad5a atm* double mutant displays an additive sensitivity towards MMS and growth-defects associated with increased cell death in the root meristem.

The ATM protein kinase is the central regulator in response to DSBs in mammals (Bensimon *et al.*, 2011). AtATM was shown to be the Arabidopsis homolog and mutants display reduced fertility and hypersensitivity towards base methylations (Garcia *et al.*, 2003). In order to define the relationship between RAD5A and the ATM-mediated DNA damage response, we created the *rad5a-2 atm-4* double mutant.

The double mutant was characterized with regards to its sensitivity against MMS-mediated base methylations and interstrand and intrastrand CLs induced by MMC and cisplatin, respectively. Therefore, we determined the dry weight of the double mutant, the respective single mutants and the WT using sensitivity assays as described. The *atm-4* single mutant did not exhibit hypersensitivity towards DNA CLs induced by MMC and cisplatin (Supplemental Figure S5). The *rad5a-2 atm-4* double mutant showed comparable sensitivities to the *rad5a-2* single mutant for both CL inducing agents. However, the induction of base methylations by MMS led to hypersensitivity of both single mutants on a comparable level (Figure 6a, b). The double mutant displayed a sensitivity that was significantly below the level of both single mutants.

Interestingly, the *rad5a-2 atm-4* double mutant exhibited a constricted growth phenotype in comparison to the WT and the respective single mutants. After at least 35 days of growth (Figure 7), double mutants featured limited growth of shoots and siliques as well as a prolonged maturation time of seeds.

As the growth defect became apparent without the application of any genotoxins, we speculated that under standard growth conditions the lack of proper replicative damage repair is indeed responsible for the phenotype. Therefore, the involvement of RAD5A and ATM in replication-associated DNA repair in dividing cells was investigated. Due to its high division activity, the root meristem is the most suitable system for the analysis of spontaneous replication stress in plants. When DNA damage leads to a block in replication, cell cycle can be arrested and cell division is inhibited, ultimately resulting in cell death. For the analysis of cell death in the root meristem, propidium iodide staining was performed as described (Curtis and Hays, 2007; Recker *et al.*, 2014). We determined the frequency of roots showing at least one dead vascular stem cell (SC), endodermal SC, epidermal SC, columella SC or transiently amplifying (TA) cell in the wild type (WT), both single mutants and the *rad5a-2 atm-4* double mutant (Figure 8). WT and both single mutants exhibited a comparable frequency of roots with dead TA cells (10 - 17 %), but no dead SCs. Interestingly, in the *rad5a-2 atm-4* double mutant, dead TA cells could be detected in about 50 % of all roots. Furthermore, the double mutant was the only line where dead SCs were found and this was with a frequency of almost 30 %. Thus, RAD5A is especially important for DNA replication in the absence of a proper DNA damage response.

DISCUSSION

In human cells, the AtRAD5A homologues HLTF and SHPRH are important DNA repair factors, the depletion of which enhances DNA damage sensitivity and promotes large chromosomal rearrangements. Furthermore, both paralogs have been associated with diverse forms of cancer and therefore represent potential tumor suppressor proteins. Both have been shown to promote PCNA polyubiquitination *in vivo* and HLTF possesses replication fork regressing activity (reviewed in Unk *et al.*, 2010). Surprisingly, little is known regarding their interaction with proteins of other repair pathways.

We previously reported the hypersensitivity of *rad5a* mutants towards the DNA CL-inducing agents MMC and cisplatin and the base-methylating genotoxin MMS (Chen *et al.*, 2008). Moreover, the At*rad5a* mutant has a defect in the conservative homologous recombination SDSA pathway, but not in single-strand annealing (SSA) (Mannuss *et al.*, 2010). As a result, AtRAD5A seems to be involved in different kinds of DNA repair pathways in plants. In the present communication, we concentrated our efforts to define the detailed roles of RAD5A in the repair of methylated bases and DNA cross-links. On the one hand, we wanted to find out whether the ATPase and RING domains in RAD5A are required for the repair of these different kinds of DNA damage. On the other hand, we aimed to define if RAD5A is interconnected with other known DNA repair factors during its actions. Mutants of different kinds of repair proteins that are involved in either one or the other or both pathways have been characterized in Arabidopsis. Thus, DNA damage induced by methylating or cross-linking agents is repaired by discrete pathways with partially common actors.

The helicase and RING domains of RAD5A are both essential for the repair of CL as well as methylated bases

ScRad5 homologs are involved in the repair of different kinds of DNA damage with strong evidence suggesting that they are involved in the error-free branch of the two-branch PRR, both in Arabidopsis and in yeast (Wang *et al.*, 2011). Different findings indicated that the RING domain of ScRad5 is involved in the polyubiquitination of PCNA, inducing the error-free PRR branch and therefore representing the regulatory involvement of ScRad5 (Hoege *et al.*, 2002; Ulrich, 2003). The DNA-processing activity of ScRad5 can be deduced from its helicase domain, as it has been shown to mediate the protein's fork regressing activity *in vitro* (Blastyak *et al.*, 2007).

In a previous study, we were already able to show that the conserved HIRAN domain of Arabidopsis RAD5A, which is involved in binding DNA repair intermediates, is essential for DNA repair *in planta* (Kobbe *et al.*, 2016). In the present study, we addressed the question as to whether both the RING and the helicase domain of Arabidopsis RAD5A are also essential for DNA repair, in general, or whether a specific domain is especially important for the repair of a particular kind of damage. By expressing different RAD5A variants in the *rad5a-2* mutant and analyzing the sensitivity towards MMC, cisplatin and MMS, we could show that both domains are indeed essential for the repair of all tested kinds of damage induced in these experiments. Thus, both enzyme activities seem to be required either simultaneously or in a sequential manner. As we assume that the enzyme functions are conserved between mammals, yeast and plants during evolution, the simplest explanation would be that the polyubiquitination of PCNA by the RING domain is a prerequisite for the helicase action of RAD5A. Hence, either the helicase function can be activated directly by a conformational change due to the polyubiquitination activity or the helicase domain is

brought to the site of action via the RAD5A mediated polyubiquitination of the target protein. The fact that the ATPase motif is conserved during evolution of all Rad5 homologs and the respective activity has been demonstrated for all homologs tested including plants, can be taken as a strong argument that the helicase-like activity in RAD5A is indeed important for the processing of DNA repair intermediates either by strand switching or fork regression. As we have shown before, the helicase AtRECQ4A is able to perform fork regression *in vitro* (Schröpfer *et al.*, 2014b) and acts in a parallel pathway to the translocase AtRAD5A in DNA repair *in vivo* (Mannuss *et al.*, 2010). We are therefore tempted to speculate that AtRAD5A is involved in a kind of strand switch repair mechanism. This is also in accordance with our recent biochemical analysis that showed that the protein is able to catalyze DNA fork regression and branch migration of Holliday junctions (Kobbe *et al.*, 2016). However, we cannot exclude that the helicase function of RAD5A is dispensable for DNA repair and it is rather the ATPase function that is necessary for the regulatory functions by facilitating PCNA polyubiquitination. Surprisingly, it was postulated that ScRad5 helicase activity is dispensable for error-free PRR and the helicase domain is rather required for interaction with Ubc13 and PCNA polyubiquitination (Ball *et al.*, 2014). Recently, this finding has been defined more precisely by showing that the ATPase function of Rad5 is necessary for interaction with the E2 complex (Choi *et al.*, 2015).

RAD5A and RAD1 do not share common pathways in DNA repair

RAD1 is a prominent factor of excision repair and forms an active nuclease together with RAD10, that is required for the incision step after recognition of the damage (Fidantsef *et al.*, 2000). In response to MMC-, cisplatin- and MMS-induced DNA damage, the *rad5a-2*

rad1-1 double mutant displayed an additive sensitivity, allowing us to conclude that both factors act in parallel pathways. Interestingly, even at very low concentrations of the CL-inducing agents, the *rad1-1* mutant displayed severe sensitivity and higher concentrations had nearly lethal effects. Reminiscent of the situation that has been described using *Xenopus* egg extract (Klein Douwel *et al.*, 2014), this might be due to the fact that plant RAD1 might also be involved in the initial incision during the process of "unhooking" an interstrand CL, subsequently enabling lesion bypass and HR. This incision represents an initial step in the mechanism of replication-coupled interstrand CL repair and the involvement of RAD1 would explain the lethal effect at higher concentrations of MMC. The repair of intrastrand CLs formed by cisplatin is most likely conducted by NER involving the endonuclease activity of the RAD1/ERCC1 heterodimer. Even though the preferred repair pathway for methylated bases induced by MMS might be BER, to a small percentage these lesions could also be repaired via NER, explaining the respective sensitivity of *rad1-1*. Furthermore, base methylations can lead to strand breaks (Wyatt and Pittman, 2006) that are possibly repaired via HR. AtRAD1 has been shown to be involved in single-strand annealing (SSA) in the presence of non-homologous overhangs (Dubest *et al.*, 2002), which might also be initiated by MMS-induced DNA damage. In contrast to this, we were previously able to show that AtRAD5A is not involved in SSA but only in SDSA in HR (Mannuss *et al.*, 2010).

Thus, as indicated by the additive sensitivity of *rad5a-2 rad1-1* that was observed in response to every genotoxin tested, we were able to show that all DNA repair sub-pathways, in which RAD5A and RAD1 are acting regarding HR, PRR and NER, are separate. Formally, we cannot exclude that RAD5A is involved in a RAD1-dependent repair pathway, as well as having an independent function to that of RAD1. Additionally, this also applies to

the following analysis addressing alternative DNA repair factors. However, using Ockham's razor we regard this possibility as unlikely, especially as such an involvement has not been reported for any other eukaryotes.

RAD5A and PARP1 act in different pathways of base methylation repair

PARPs catalyze the synthesis and transfer of poly ADP-ribose (PAR) to target proteins and the mammalian ortholog of AtPARP1 is a prominent factor in SSB repair and BER (reviewed in Gibson and Kraus, 2012). At*parp1* mutants are sensitive towards MMS, indicating a likely conservation of function among animals and plants (Boltz *et al.*, 2014). We were able to reproduce the sensitivity of *parp1-1* towards MMS-induced DNA damage, while we could not detect any hypersensitivity against the CL-inducing agents MMC or cisplatin. This indicates that AtPARP1 is part of one or several pathways that are able to cope with methylated bases but not CLs. We observed an additive hypersensitivity of the *rad5a-2 parp1-1* double mutant in response to MMS. However, no synergies of PARP1 and RAD5A in response to DNA CLs were revealed as the *rad5a-2 parp1-1* double mutant displayed the same level of sensitivity as the *rad5a-2* single mutant. This further strengthens our conclusion that PARP1 is only involved in the repair of base methylations, but not in the repair of CLs. Furthermore, RAD5A and PARP1 act in separate pathways in response to MMS induced DNA damage. Considering the involvement of RAD5A in PRR, it is tempting to argue that the sensitivity of *parp1-1* is because of a function of PARP1 in BER and SSBR. Numerous findings indicate that HsPARP1 ensures HR-independent repair of strand breaks as HsBRCA1/2-deficient tumor cells are highly sensitive towards PARP-inhibitors (reviewed in Lee *et al.*, 2014). Assuming that these functions are conserved for AtPARP1 as well, this

illustrates the sensitivity of *parp1-1* towards MMS, as unremoved base methylations can lead to strand breaks (Wyatt and Pittman, 2006). In summary, we were able to show that AtPARP1 is involved in the repair of MMS-induced DNA damage in a pathway independent from PRR, which possibly corresponds to BER and HR-independent SSBR.

RAD5A and TEB have independent roles in repair of DNA cross-links and base methylations

AtTEB has been described as the homolog of Drosophila MUS308 and mammalian DNA polymerase θ and has been successfully linked to DNA repair, cell differentiation, cell cycle control and progression of replication (Inagaki *et al.*, 2006; Inagaki *et al.*, 2009). We were able to reproduce the sensitivity of *teb-5* towards MMS and MMC and could further demonstrate a hypersensitivity towards the intrastrand CL-inducing genotoxin cisplatin. By testing different concentrations of the respective genotoxins, we could show that the *rad5a-2 teb-5* double mutant displays an additive sensitivity towards all different types of DNA damage induced. This reveals that different DNA repair pathways are affected and RAD5A and TEB act on different substrates during the repair of CLs, as well as base methylations. Due to the fasciated phenotype of the mutant, TEB has been linked to replicative DNA repair. Additionally, it has been observed that in absence of the HR-related factors XRCC2 and RAD51D, the developmental phenotype of *teb* is further enhanced (Inagaki *et al.*, 2009). These strong genetic interactions suggest that TEB is involved in safe guarding the progression of DNA replication. Since HR represents a pathway to recover from stress that perturbs replication (Lambert *et al.*, 2007), these results indicate that TEB acts parallel to HR in restarting replication blocks. A function of the polymerase θ in the alternative NHEJ

pathway could be demonstrated recently, in various multicellular eukaryotes (Roerink *et al.*, 2014; Ceccaldi *et al.*, 2015; Kent *et al.*, 2015; Mateos-Gomez *et al.*, 2015; van Schendel *et al.*, 2015). Thus, in the mutant, DSBs induced by genotoxic treatments cannot be repaired as efficiently as in the presence of the enzyme. Furthermore, it is possible that TEB acts as a polymerase within TLS, which has been shown for the human homolog POLθ (Yoon *et al.*, 2014). This function would play a role within the TLS branch of PRR in response to MMS and cisplatin and the repair of interstrand CL, as TLS is required to close the gap after the process of unhooking the CL. Thus, although for the moment we are not able to narrow down all specific functions of TEB, the additive sensitivity of *rad5a-2 teb-5* towards various types of DNA damage clearly demonstrates that both proteins are definitely involved in different pathways.

RAD5A is able to partly complement an ATM-deficient DNA damage response

In mammals, the protein kinase ATM is the initial factor in response to DNA damage (particularly DSBs) and phosphorylates target proteins in the following activation of the repair machinery (Shiloh, 2001). These functions, and its role in expression regulation of DNA repair factors following DSBs, were confirmed in the Arabidopsis homolog (Garcia *et al.*, 2003). It was reported before that At*atm* mutants are sensitive towards MMS and display reduced fertility, attributable to abundant chromosomal fragmentation during meiosis (Garcia *et al.*, 2003). There was no hypersensitivity of the *atm-4* mutant detectable against the CL-inducing agents cisplatin or MMC and no synergies of *rad5a-2 atm-4*, whereas we were able to reproduce the sensitivity of *atm-4* towards MMS induced base methylations. During the repair of base methylations via BER, abasic sites are produced

which lead to an inhibition of DNA replication and can indirectly cause DSBs (Menke *et al.*, 2001). Therefore, ATM seems to be indirectly involved in the repair of DSBs following MMS treatment. The additive sensitivity of the *rad5a-2 atm-4* double mutant indicates that RAD5A and ATM can partly complement each other during the repair of base methylations and act in independent pathways. Results from human cell culture support these findings as the knockdown of ATM did not result in a decrease of mono- and polyubiquitinated PCNA (Motegi *et al.*, 2008). Interestingly, the *rad5a-2 atm-4* double mutants displayed a growth restricted phenotype without MMS treatment that further substantiates the independent roles of RAD5A and ATM also in response to spontaneous DNA damage. Due to the absence of RAD5A and ATM, endogenous replicative DNA damage accumulates, leading to an increased cell death. This could be confirmed by the analysis of the fast dividing root meristems. The *rad5a-2 atm-4* double mutant exhibited an increased number of dead TA and stem cells compared to both single mutants. Thus, RAD5A is able to partly complement for a deficient DNA damage response. It is tempting to speculate which specific functions of both proteins are responsible for the phenomenon. Loss of ATM results in a loss of induced expression of critical components of the HR pathway, such as AtBRCA1 and AtRAD51 (Culligan *et al.*, 2006), in addition to other deficiencies . We could show before that the loss of RAD5A is correlated with deficiencies in certain HR reactions (Chen *et al.*, 2008; Mannuss *et al.*, 2010). As a result, it might well be that the accumulation of replication-associated DNA damage that would be normally processed by HR pathways might cause enhanced cell death and ultimately growth retardation. Indeed RAD5A might be involved in a specific branch of HR that is at least partly independent from ATM-control. Our findings are supported with results from phosphoproteome and transcriptome analyses, whereby RAD5A was neither found as a target for ATM-mediated phosphorylation nor was any

influence of ATM on RAD5A expression detected (Ricaud *et al.*, 2007; Roitinger *et al.*, 2015). The expression of PARP1 was shown to be ATM-regulated (Garcia *et al.*, 2003), thus further emphasizing possible repair pathways with ATM and PARP1 in parallel to RAD5A.

Our study demonstrates that RAD5A, during DNA repair, has independent functions in relation to key factors of other prominent DNA repair pathways in plants. Nevertheless, this does not imply the protein is able to process DNA repair reactions on its own. Besides factors that are also involved in general DNA replication, further analysis may well identify further DNA processing factors specifically involved in the error free branch of post-replicative repair in plants.

EXPERIMENTAL PROCEDURES

Plant lines and plant growth conditions

The mutants lines *rad5a-2* [SALK_047150 (Chen *et al.*, 2008)], *parp1-1* [GABI_692A05 (Jia *et al.*, 2013)], *rad1-1* [SALK_096156 (Yoshiyama *et al.*, 2009)], *teb-5* [SALK_018851 (Inagaki *et al.*, 2006)] and *atm-4* [SALK_036940 (Inagaki *et al.*, 2009)] have been previously described. To generate the analyzed double mutants, respective homozygous plants were crossed, and the homozygous double mutants were identified in the F2 progeny by PCR-based genotyping. Details on genotyping are given in Supplemental table S1. For reproduction of the plant lines, plants were grown in soil (1:1 mixture of Floraton 3 [Floragard, Oldenburg, Germany] and vermiculite [1-3 mm; Deutsche Vermiculite Dämmstoff GmbH, Sprockhövel, Germany]) under long day conditions (16 h light/8 h dark) at 22 °C. For axenic plant culture, seeds were surface sterilized with 6 % sodium hypochlorite solution and rinsed in ddH_2O.

After stratification overnight at 4 °C, surface sterilized seeds were plated on germination medium (GM: 4.9 g/l Murashige & Skoog-medium, 10 g/l saccharose, pH 5.7, 7.6 g/l agar) supplemented with or without genotoxins and incubated in a plant growth chamber (Percival Scientific, CU-36L4; 16 h light at 22 °C/8 h dark at 20 °C).

Cloning of *RAD5A* constructs for plant transformation

All vectors containing different *RAD5A* constructs were based on the binary plasmid pPZP221 for plant transformation (Hajdukiewicz *et al.*, 1994). All *RAD5A* constructs included a promoter region, a coding region amplified from *A. thaliana* Col-0 WT gDNA (5498 bp) and a terminator region. The promoter (763 bp upstream of the start codon of *RAD5A*) and terminator (518 bp downstream of the stop codon of *RAD5A*) sequences were amplified from genomic DNA. Furthermore, the constructs *RAD5A-HD* (KT425/426AA) and *RAD5A-RD* (C794S), which contain point mutations leading to the respective amino acid substitutions, were cloned. Detailed information about the cloning PCRs, primer combinations, primer sequences and PCR templates is given in Supplemental table S2. The integrity of all pPZP221 derivatives was verified by sequencing (GATC Biotech AG, Konstanz).

Generation of transgenic plant lines

Using the GV3101::pMP90 *Agrobacterium* strain (Koncz *et al.*, 1984), the pPZP221-based *RAD5A* constructs were transformed into Arabidopsis plants via the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Transgenic plants containing the transformed T-DNA were selected by plating on solid GM selection medium (60 mg/l

gentamycin) in T1. To identify single locus lines in generation T2, statistical analyses [critical value χ^2 (1;0.95)] of the segregation behavior of the plant lines were performed. Homozygous T-DNA containing plants were selected on selection medium in generation T3 and subsequently used in assays.

Sensitivity assays

Arabidopsis seeds were surface sterilized with 6 % sodium hypochlorite solution, rinsed in ddH_2O and stratified overnight at 4 °C. Sterilized seeds were plated on pure or genotoxic agent-containing germination medium (MMS and cisplatin, Sigma-Aldrich, Taufenkirchen, Germany; MMC, Duchefa Biochemie, Haarlem, Netherlands). After 22 d in a plant growth chamber the effects of the individual genotoxins on plant growth were evaluated. The plants were dried overnight and the dry weight of treated seedlings was normalized to that of untreated controls of the same line. For every line, at least 24 plants per assay were analysed. Each assay was performed at least three times and the mean values including the standard deviations were determined. For statistical analysis, a two-tailed t-test with unequal variances was performed and p-values were calculated.

Cell death analysis in roots

Arabidopsis seeds were treated as mentioned above and sown on germination medium. After 4 d incubation in a plant growth chamber, the plants were transferred to 6-well plates containing 5 ml liquid germination medium per well. After a further incubation of 24 h, the plantlets were transferred into 100 µl propidium iodide solution (5 µg/ml) on microscope

slides and covered with a coverslip. Microscopy was performed with a LSM 700 laser scanning microscope (Carl Zeiss Microscopy, Göttingen, Germany).

ACCESSION NUMBERS

Sequence data from this article can be found with the following Arabidopsis AGI locus identifiers, At*RAD1*: At5g41150, At*PARP1*: At2g31320, At*RAD5A*: At5g22750, At*TEB*: At4g32700, At*ATM*: At3g48190

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The authors declare no conflict of interest.

SUPPORTING INFORMATION

Figure S1. Wild type (WT) lines transformed with the full length *RAD5A*, *RAD5A-HD* and *RAD5A-RD* construct.

Figure S2. Gene expression analysis of the *rad5a-2* complementation lines.

Figure S3. Western blot analysis of complemented *rad5a-2* mutant plants.

Figure S4. The role of RAD5A and PARP1 in response to MMC and cisplatin-induced DNA damage.

Figure S5. The role of RAD5A and ATM in response to MMC and cisplatin-induced DNA damage.

Table S1. Primers used for PCR-based genotyping

Table S2. Cloning PCRs and primers used to create plasmids for in vivo studies.

REFERENCES

Aboussekhra, A. and Wood, R.D. (1994) Repair of UV-damaged DNA by mammalian cells and Saccharomyces cerevisiae. *Current opinion in genetics & development*, 4, 212–220.

Amor, Y., Babiychuk, E., Inze, D. and Levine, A. (1998) The involvement of poly(ADP-ribose) polymerase in the oxidative stress responses in plants. *FEBS letters*, 440, 1–7.

Ball, L.G., Xu, X., Blackwell, S., Hanna, M.D., Lambrecht, A.D. and Xiao, W. (2014) The Rad5 helicase activity is dispensable for error-free DNA post-replication repair. *DNA repair*, 16, 74–83.

Barbour, L. and Xiao, W. (2003) Regulation of alternative replication bypass pathways at stalled replication forks and its effects on genome stability: a yeast model. *Mutation research*, 532, 137–155.

Bensimon, A., Aebersold, R. and Shiloh, Y. (2011) Beyond ATM. the protein kinase landscape of the DNA damage response. *FEBS letters*, 585, 1625–1639.

Blastyak, A., Pinter, L., Unk, I., Prakash, L., Prakash, S. and Haracska, L. (2007) Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression. *Molecular cell*, 28, 167–175.

Boltz, K.A., Jasti, M., Townley, J.M. and Shippen, D.E. (2014) Analysis of poly(ADP-Ribose) polymerases in Arabidopsis telomere biology. *PloS one*, 9, e88872.

Boyd, J.B., Sakaguchi, K. and Harris, P.V. (1990) mus308 mutants of Drosophila exhibit hypersensitivity to DNA cross-linking agents and are defective in a deoxyribonuclease. *Genetics*, 125, 813–819.

Broomfield, S., Hryciw, T. and Xiao, W. (2001) DNA postreplication repair and mutagenesis in Saccharomyces cerevisiae. *Mutation research*, 486, 167–184.

Ceccaldi, R., Liu, J.C., Amunugama, R., Hajdu, I., Primack, B., Petalcorin, M.I., O'Connor, K.W., Konstantinopoulos, P.A., Elledge, S.J., Boulton, S.J., Yusufzai, T. and D'Andrea, A.D. (2015) Homologous-recombination-deficient tumours are dependent on Poltheta-mediated repair. *Nature*, 518, 258–262.

Chen, I.P., Mannuss, A., Orel, N., Heitzeberg, F. and Puchta, H. (2008) A homolog of ScRAD5 is involved in DNA repair and homologous recombination in Arabidopsis. *Plant physiology*, 146, 1786–1796.

Choi, K., Batke, S., Szakal, B., Lowther, J., Hao, F., Sarangi, P., Branzei, D., Ulrich, H.D. and Zhao, X. (2015) Concerted and differential actions of two enzymatic domains underlie Rad5 contributions to DNA damage tolerance. *Nucleic acids research*, 43, 2666–2677.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *The Plant journal : for cell and molecular biology*, 16, 735–743.

Culligan, K., Tissier, A. and Britt, A. (2004) ATR regulates a G2-phase cell-cycle checkpoint in Arabidopsis thaliana. *The Plant cell*, 16, 1091–1104.

Culligan, K.M. and Britt, A.B. (2008) Both ATM and ATR promote the efficient and accurate processing of programmed meiotic double-strand breaks. *The Plant journal : for cell and molecular biology*, 55, 629–638.

Culligan, K.M., Robertson, C.E., Foreman, J., Doerner, P. and Britt, A.B. (2006) ATR and ATM play both distinct and additive roles in response to ionizing radiation. *The Plant journal : for cell and molecular biology*, 48, 947–961.

Curtis, M.J. and Hays, J.B. (2007) Tolerance of dividing cells to replication stress in UVB-irradiated Arabidopsis roots: requirements for DNA translesion polymerases eta and zeta. *DNA repair*, 6, 1341–1358.

de Block, M., Verduyn, C., de Brouwer, D. and Cornelissen, M. (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *The Plant journal : for cell and molecular biology*, 41, 95–106.

de Laat, W.L., Hoeijmakers, J.H. and Jaspers, N.G. (1999) Molecular mechanism of nucleotide excision repair. *Genes & development*, 13, 768–785.

Dubest, S., Gallego, M.E. and White, C.I. (2002) Role of the AtRad1p endonuclease in homologous recombination in plants. *EMBO reports*, 3, 1049–1054.

Eastman, A. (1985) Interstrand cross-links and sequence specificity in the reaction of cis-dichloro(ethylenediamine)platinum(II) with DNA. *Biochemistry*, 24, 5027–5032.

Fidantsef, A.L., Mitchell, D.L. and Britt, A.B. (2000) The Arabidopsis UVH1 gene is a homolog of the yeast repair endonuclease RAD1. *Plant physiology*, 124, 579–586.

Flynn, R.L. and Zou, L. (2011) ATR: a master conductor of cellular responses to DNA replication stress. *Trends in biochemical sciences*, 36, 133–140.

Gallego, F., Fleck, O., Li, A., Wyrzykowska, J. and Tinland, B. (2000) AtRAD1, a plant homologue of human and yeast nucleotide excision repair endonucleases, is involved in

dark repair of UV damages and recombination. *The Plant journal : for cell and molecular biology*, 21, 507–518.

Garcia, V., Bruchet, H., Camescasse, D., Granier, F., Bouchez, D. and Tissier, A. (2003) AtATM is essential for meiosis and the somatic response to DNA damage in plants. *The Plant cell*, 15, 119–132.

Gibson, B.A. and Kraus, W.L. (2012) New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nature reviews. Molecular cell biology*, 13, 411–424.

Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant molecular biology*, 25, 989–994.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, 419, 135–141.

Inagaki, S., Nakamura, K. and Morikami, A. (2009) A link among DNA replication, recombination, and gene expression revealed by genetic and genomic analysis of TEBICHI gene of Arabidopsis thaliana. *PLoS genetics*, 5, e1000613.

Inagaki, S., Suzuki, T., Ohto, M.A., Urawa, H., Horiuchi, T., Nakamura, K. and Morikami, A. (2006) Arabidopsis TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. *The Plant cell*, 18, 879–892.

Jia, Q., den Dulk-Ras, A., Shen, H., Hooykaas, P.J. and de Pater, S. (2013) Poly(ADP-ribose)polymerases are involved in microhomology mediated back-up non-homologous end joining in Arabidopsis thaliana. *Plant molecular biology*, 82, 339–351.

Kent, T., Chandramouly, G., McDevitt, S.M., Ozdemir, A.Y. and Pomerantz, R.T. (2015) Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase theta. *Nature structural & molecular biology*, 22, 230–237.

Klein Douwel, D., Boonen, R.A., Long, D.T., Szypowska, A.A., Raschle, M., Walter, J.C. and Knipscheer, P. (2014) XPF-ERCC1 acts in Unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. *Molecular cell*, 54, 460–471.

Kobbe, D., Kahles, A., Walter, M., Klemm, T., Mannuss, A., Knoll, A., Focke, M. and Puchta, H. (2016) AtRAD5A is a DNA translocase harboring a HIRAN domain which confers binding to branched DNA structures and is required for DNA repair in vivo. *The Plant journal : for cell and molecular biology*, 88, 521–530.

Kobbe, S., Trapp, O., Knoll, A., Manuss, A. and Puchta, H. (2015) The Translesion Polymerase ζ Has Roles Dependent on and Independent of the Nuclease MUS81 and the Helicase RECQ4A in DNA Damage Repair in Arabidopsis. *Plant physiology*, 169, 2718–2729.

Koncz, C., Kreuzaler, F., Kalman, Z. and Schell, J. (1984) A simple method to transfer, integrate and study expression of foreign genes, such as chicken ovalbumin and alpha-actin in plant tumors. *The EMBO journal*, 3, 1029–1037.

Lambert, S., Froget, B. and Carr, A.M. (2007) Arrested replication fork processing. interplay between checkpoints and recombination. *DNA repair*, 6, 1042–1061.

Lee, J.M., Ledermann, J.A. and Kohn, E.C. (2014) PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 25, 32–40.

Lin, J.R., Zeman, M.K., Chen, J.Y., Yee, M.C. and Cimprich, K.A. (2011) SHPRH and HLTF act in a damage-specific manner to coordinate different forms of postreplication repair and prevent mutagenesis. *Molecular cell*, 42, 237–249.

Liu, Z., Hossain, G.S., Islas-Osuna, M.A., Mitchell, D.L. and Mount, D.W. (2000) Repair of UV damage in plants by nucleotide excision repair. Arabidopsis UVH1 DNA repair gene is a homolog of Saccharomyces cerevisiae Rad1. *The Plant journal : for cell and molecular biology*, 21, 519–528.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S. and Weissman, A.M. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 11364–11369.

Mannuss, A., Dukowic-Schulze, S., Suer, S., Hartung, F., Pacher, M. and Puchta, H. (2010) RAD5A, RECQ4A, and MUS81 have specific functions in homologous recombination and define different pathways of DNA repair in Arabidopsis thaliana. *The Plant cell*, 22, 3318–3330.

Masaoka, A., Horton, J.K., Beard, W.A. and Wilson, S.H. (2009) DNA polymerase beta and PARP activities in base excision repair in living cells. *DNA repair*, 8, 1290–1299.

Mateos-Gomez, P.A., Gong, F., Nair, N., Miller, K.M., Lazzerini-Denchi, E. and Sfeir, A. (2015) Mammalian polymerase theta promotes alternative NHEJ and suppresses recombination. *Nature*, 518, 254–257.

Menke, M., Chen, I., Angelis, K.J. and Schubert, I. (2001) DNA damage and repair in Arabidopsis thaliana as measured by the comet assay after treatment with different classes of genotoxins. *Mutation research*, 493, 87–93.

Motegi, A., Liaw, H.J., Lee, K.Y., Roest, H.P., Maas, A., Wu, X., Moinova, H., Markowitz, S.D., Ding, H., Hoeijmakers, J.H. and Myung, K. (2008) Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication

forks. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 12411–12416.

Muzzini, D.M., Plevani, P., Boulton, S.J., Cassata, G. and Marini, F. (2008) Caenorhabditis elegans POLQ-1 and HEL-308 function in two distinct DNA interstrand cross-link repair pathways. *DNA repair*, 7, 941–950.

Pegg, A.E. (1984) Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer investigation*, 2, 223–231.

Recker, J., Knoll, A. and Puchta, H. (2014) The Arabidopsis thaliana homolog of the helicase RTEL1 plays multiple roles in preserving genome stability. *The Plant cell*, 26, 4889–4902.

Ricaud, L., Proux, C., Renou, J.-P., Pichon, O., Fochesato, S., Ortet, P. and Montane, M.-H. (2007) ATM-mediated transcriptional and developmental responses to gamma-rays in Arabidopsis. *PloS one*, 2, e430.

Richmond, E. and Peterson, C.L. (1996) Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2. *Nucleic acids research*, 24, 3685–3692.

Rink, S.M., Lipman, R., Alley, S.C., Hopkins, P.B. and Tomasz, M. (1996) Bending of DNA by the mitomycin C-induced, GpG intrastrand cross-link. *Chemical research in toxicology*, 9, 382–389.

Roerink, S.F., van Schendel, R. and Tijsterman, M. (2014) Polymerase theta-mediated end joining of replication-associated DNA breaks in C. elegans. *Genome research*, 24, 954–962.

Roitinger, E., Hofer, M., Kocher, T., Pichler, P., Novatchkova, M., Yang, J., Schlögelhofer, P. and Mechtler, K. (2015) Quantitative phosphoproteomics of the ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia-mutated and rad3-related (ATR) dependent DNA damage response in Arabidopsis thaliana. *Molecular & cellular proteomics : MCP*, 14, 556–571.

Sakamoto, A., Lan, V.T., Hase, Y., Shikazono, N., Matsunaga, T. and Tanaka, A. (2003) Disruption of the AtREV3 gene causes hypersensitivity to ultraviolet B light and gamma-rays in Arabidopsis. implication of the presence of a translesion synthesis mechanism in plants. *The Plant cell*, 15, 2042–2057.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S.R., Simmons, A., Clines, G.A., Sartiel, A., Gatti, R.A., Chessa, L., Sanal, O., Lavin, M.F., Jaspers, N.G., Taylor, A.M., Arlett, C.F., Miki, T., Weissman, S.M., Lovett, M., Collins, F.S. and Shiloh, Y. (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science (New York, N.Y.)*, 268, 1749–1753.

Schreiber, V., Ame, J.C., Dolle, P., Schultz, I., Rinaldi, B., Fraulob, V., Menissier-De Murcia, J. and de Murcia, G. (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. *The Journal of biological chemistry*, 277, 23028–23036.

Schröpfer, S., Knoll, A., Trapp, O. and Puchta, H. (2014a) Nucleus and Genome. DNA Recombination and Repair. In *Molecular Biology (*Howell, S.H., ed): Springer New York, pp. 1–37.

Schröpfer, S., Kobbe, D., Hartung, F., Knoll, A. and Puchta, H. (2014b) Defining the roles of the N-terminal region and the helicase activity of RECQ4A in DNA repair and homologous recombination in Arabidopsis. *Nucleic acids research*, 42, 1684–1697.

Shiloh, Y. (2001) ATM and ATR: networking cellular responses to DNA damage. *Current opinion in genetics & development*, 11, 71–77.

Ukai, A., Maruyama, T., Mochizuki, S., Ouchida, R., Masuda, K., Kawamura, K., Tagawa, M., Kinoshita, K., Sakamoto, A., Tokuhisa, T. and J, O.W. (2006) Role of DNA polymerase

theta in tolerance of endogenous and exogenous DNA damage in mouse B cells. *Genes to cells : devoted to molecular & cellular mechanisms*, 11, 111–121.

Ulrich, H.D. (2003) Protein-protein interactions within an E2-RING finger complex. Implications for ubiquitin-dependent DNA damage repair. *The Journal of biological chemistry*, 278, 7051–7058.

Ulrich, H.D. and Jentsch, S. (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. *The EMBO journal*, 19, 3388–3397.

Unk, I., Hajdu, I., Blastyak, A. and Haracska, L. (2010) Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance. *DNA repair*, 9, 257–267.

Unk, I., Hajdu, I., Fatyol, K., Hurwitz, J., Yoon, J.H., Prakash, L., Prakash, S. and Haracska, L. (2008) Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 3768–3773.

Unk, I., Hajdu, I., Fatyol, K., Szakal, B., Blastyak, A., Bermudez, V., Hurwitz, J., Prakash, L., Prakash, S. and Haracska, L. (2006) Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 18107–18112.

van Kregten, M., de Pater, S., Romeijn, R., van Schendel, R., Hooykaas, P.J.J. and Tijsterman, M. (2016) T-DNA integration in plants results from polymerase-theta-mediated DNA repair. *Nature plants*, 2, 16164.

van Schendel, R., Roerink, S.F., Portegijs, V., van den Heuvel, S. and Tijsterman, M. (2015) Polymerase Theta is a key driver of genome evolution and of CRISPR/Cas9-mediated mutagenesis. *Nature communications*, 6, 7394.

Vanderauwera, S., de Block, M., van de Steene, N., van de Cotte, B., Metzlaff, M. and van Breusegem, F. (2007) Silencing of poly(ADP-ribose) polymerase in plants alters abiotic stress signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 15150–15155.

Wang, S., Wen, R., Shi, X., Lambrecht, A., Wang, H. and Xiao, W. (2011) RAD5a and REV3 function in two alternative pathways of DNA-damage tolerance in Arabidopsis. *DNA repair*, 10, 620–628.

Woodhouse, B.C. and Dianov, G.L. (2008) Poly ADP-ribose polymerase-1: an international molecule of mystery. *DNA repair*, 7, 1077–1086.

Wyatt, M.D. and Pittman, D.L. (2006) Methylating agents and DNA repair responses. Methylated bases and sources of strand breaks. *Chemical research in toxicology*, 19, 1580–1594.

Yoon, J.H., Roy Choudhury, J., Park, J., Prakash, S. and Prakash, L. (2014) A role for DNA polymerase theta in promoting replication through oxidative DNA lesion, thymine glycol, in human cells. *The Journal of biological chemistry*, 289, 13177–13185.

Yoshiyama, K., Conklin, P.A., Huefner, N.D. and Britt, A.B. (2009) Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 12843–12848.

FIGURE LEGENDS

Figure 1. Schematic representation of the recombinant RAD5A variants. The respective *RAD5A* constructs that were transformed into *rad5a-2* mutants are depicted. RAD5A-HD

contains amino acid substitutions (red box, KT425/426AA, lysine and threonine to alanine and alanine at positions 425/426) in the Walker A motif of the helicase domain. RAD5A-RD contains an amino acid substitution (red box, C794S, cysteine to alanine at position 794), affecting a cysteine involved in Zn^{2+} coordination in the RING domain (blue).

Figure 2. The role of the RING and the helicase domain of RAD5A in response to MMC, cisplatin and MMS-induced DNA damage. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing 5 µg/ml MMC (a-c), 5 µM cisplatin (d-f) and 90 ppm MMS (g-i), was determined. The relative dry weight per plant was normalized to the dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times and the mean values including the standard deviations (error bars) are depicted. For each construct, three randomly selected independent integration lines were tested (#1-#3). Expression of the wild type construct *RAD5A* in the *rad5a-2* mutant led to full complementation of the hypersensitivity of *rad5a-2* towards MMC (a), cisplatin (d) and MMS (g). The hypersensitivity could not be complemented by the expression of the helicase domain-defective *RAD5A-HD* construct (b, e, h) or the RING domain-defective *RAD5A-RD* construct (c, f, i). *$p < 0.05$; ***$p < 0.001$

Figure 3. The role of RAD5A and RAD1 in response to MMS, MMC and cisplatin-induced DNA damage. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing MMS (a, b), MMC (c, d) and cisplatin (e, f) was determined. The relative dry weight per plant was normalized to the dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times and the mean

values including the standard deviations (error bars) are depicted. In response to MMS-induced base methylations, both single mutants exhibited a significant reduction of relative dry weight compared to the wild type (WT) (a). At 100 ppm of MMS, the *rad5a-2 rad1-1* double mutant showed an additive sensitivity with a relative dry weight that was significantly lower than that of both single mutants (b). After induction of interstrand CLs via MMC (c) and intrastrand CLs via cisplatin (e) the *rad5a-2 rad1-1* double mutant displayed a relative dry weight that was significantly lower than that of the *rad1-1* single mutant. At higher concentrations of CL-inducing agents (d, f), both single mutants showed hypersensitivity compared to the WT and the double mutant was comparable to the *rad1-1* single mutant due to an almost lethal phenotype of RAD1 deficient plant lines. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Figure 4. The role of RAD5A and PARP1 in response to MMS-induced DNA damage. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing MMS (a, b) was determined. The relative dry weight per plant was normalized to the dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times and the mean values including the standard deviations (error bars) are depicted. The single mutant lines *rad5a-2* and *parp1-1* were hypersensitive at one concentration (b) with a relative dry weight significantly below that of the wild type (WT). Both concentrations of MMS (a, b) led to an additive sensitivity of the *rad5a-2 parp1-1* double mutant, displaying a relative dry weight that was lower than that of the respective single mutants on a significant level. *$p < 0.05$; **$p < 0.01$

Figure 5. The role of RAD5A and TEB in response to MMS, MMC and cisplatin-induced DNA damage. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing MMS (a, b), MMC (c, d) and cisplatin (e, f) was determined. The relative dry weight per plant was normalized to the dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times and the mean values including the standard deviations (error bars) are depicted. In response to MMS-induced base methylations, both single mutants exhibited a significant reduction of relative dry weight compared to the wild type (WT) (a). The *rad5a-2 teb-5* double mutant displayed an additive hypersensitivity that was not detectable at higher concentrations of MMS (b). After treatment with 1.5 µg/ml MMC (c) and 1.5 µM (e) as well as 2.5 µM cisplatin (f), an additive hypersensitivity of the *rad5a-2 teb-5* double mutant could be observed, showing a relative dry weight significantly below that of the single mutants. This effect was not detectable at a higher concentration of MMC (d), due to an almost lethal phenotype of the RAD5A deficient plants. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Figure 6. The role of RAD5A and ATM in response to MMS-induced DNA damage. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing MMS (a, b), was determined. The relative dry weight per plant was normalized to the dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times and the mean values including the standard deviations (error bars) are depicted. Both single mutants showed a significant reduction of relative dry weight compared to the wild type (WT) in response to MMS-induced base methylations. The *rad5a-2 atm-4* double mutant displayed a relative dry weight significantly below both single mutants, thus indicating an additive sensitivity. *$p < 0.05$; **$p < 0.01$

Figure 7. Growth defects due to the concurrent loss of RAD5A and ATM. The growth phenotype of 35 days old plants grown in soil was determined. Both single mutants displayed vegetative growth comparable to the wild type (WT). Siliques in *atm-4* were shorter to those in the WT and *rad5a-2*. Compared to the single mutants, *rad5a-2 atm-4* double mutants exhibited restricted growth, particularly of the stem.

Figure 8. The role of RAD5A and ATM in replicative stress in the root meristem. The frequency of roots exhibiting at least one dead cell of the specified cell type was determined by propidium iodide staining (a). Each assay was performed three times and the mean values including the standard deviations (error bars) are depicted. The cells were categorized into vascular stem cells (SC), endodermal SC, epidermal SC, columella SC and transiently amplifying (TA) cells. The *rad5a-2 atm-4* double mutant showed an increased frequency of roots with dead TA cells compared to both single mutants and the wild type (WT). Dead stem cells were merely found in the double mutant, in which only vascular SCs were affected. Representative images of propidium iodide stained root tips from each line are depicted (b). Bar = 50 µm